

Regulation of Proteases after Spinal Cord Injury

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Abstract

Spinal cord injury is a major medical problem worldwide. Unfortunately, we still do not have suitable therapeutic agents for the treatment of spinal cord injury and prevention of its devastating consequences. Scientists and physicians are baffled by the challenges of controlling progressive neurodegeneration in spinal cord injury, which has not been healed with any currently-available treatments. Although extensive work has been carried out to better understand the pathophysiology of spinal cord injury, our current understanding of the repair mechanisms of secondary injury processes is still meager. Several investigators reported the crucial role played by various proteases after spinal cord injury. Understanding the beneficial and harmful roles these proteases play after spinal cord injury will allow scientists to plan and design appropriate treatment strategies to improve functional recovery after spinal cord injury. This review will focus on various proteases such as matrix metalloproteinases, cysteine proteases, and serine proteases and their inhibitors in the context of spinal cord injury.

Key words: caspase; cathepsin; matrix metalloproteinase; plasminogen activator; spinal cord injury

Introduction

TRAUMATIC SPINAL CORD INJURY (SCI) is one of the most devastating injuries to afflict the human body. Trauma resulting in SCI is a leading cause of permanent disability in young adults, resulting in partial or complete loss of motor and sensory function below the lesion site. The majority of injured patients are disabled during the most productive period of their lives. The injury is prevalent in the younger population, creating physical, emotional, and economic burdens on both the individual and society. The devastating neurological consequences of spinal cord injuries are largely attributed to retrograde neuronal cell death and failure of surviving neurons to regenerate their severed axons. Neurological consequences reflect not only the initial mechanical damage, but also the complex secondary events that promote neuronal injury, demyelination, and aberrant wound healing processes. SCI results in the initial physical disruption of structures in the spinal cord (primary injury), and in the generation of secondary events that collectively injure intact neighboring tissues (secondary injury). The secondary injury occurs over the hours and days after SCI, further intensifying tissue damage and functional deficits. Extending from days to years after the trauma, apoptotic cell death continues and scarring and demyelination accompany wallerian degeneration, ultimately leading to conduction deficits. Reducing the extent of progressive tissue loss following SCI is an essential step toward recovery after SCI. Research investigations revealed that several proteases play key roles in the secondary

pathogenesis and recovery after SCI. This review will discuss both the positive and negative roles of various proteases, such as matrix metalloproteinases, cysteine proteases (calpains, caspases, and cathepsins), and serine proteases (urokinase plasminogen activator, tissue plasminogen activator, granzymes, and neuropsin), and their inhibitors in the context of SCI. A summary of various key proteases and their roles in the context of SCI are presented in Table 1.

Matrix metalloproteinases

MMPs are a family of zinc-containing endopeptidases characterized by sequence homology and their ability to cleave extracellular matrix (ECM) proteins. These were first discovered in the involuting tadpole tail (Gross and Lapiere, 1962; Gross and Nagai, 1965). MMPs are a collection of over 28 endopeptidases that are divided according to their substrate specificity, sequence similarity, and domain organization, into collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10), matrilysins (MMP-7 and MMP-26), convertase-activable secreted MMPs (MMP-11, MMP-21, and MMP-28), convertase-activable membrane-type (MT) MMPs (MMP-14 [MT1-MMP], MMP-15 [MT2-MMP], MMP-16 [MT3-MMP], MMP-17 [MT4-MMP], MMP-24 [MT5-MMP], and MMP-25 [MT6-MMP]), and other MMPs (MMP-12, MMP-19, MMP-20, MMP-22, MMP-23A, and MMP-23B, among others); (Stamenkovic, 2000). Each MMP contains a hinge region, a hemopexin domain, an activation locus in the “pro” domain,

TABLE 1. SUMMARY OF VARIOUS KEY PROTEASES AND THEIR ROLES IN THE CONTEXT OF SPINAL CORD INJURY

Type of protease	Name of protease	Role after spinal cord injury	References
Matrix metalloproteinases	MMP-2	Implicated in wound-healing processes, including remodeling of the ECM and glial scar formation; MMP-2 knockouts showed greater CSPG immunoreactivity, fewer serotonergic fibers caudal to the injury site, and significantly reduced motor recovery	Duchossoy et al., 2001; Xu et al., 2001; Goussev et al., 2003; Hsu et al., 2006; Veeravalli et al., 2009a
	MMP-9	Contribute to the disruption of the blood–spinal cord barrier and early pathogenesis after SCI; MMP inhibitors reduced barrier disruption and apoptotic cell death; MMP-9 knockouts showed reduced barrier disruption and CSPG immunoreactivity, and abrogated glial scar formation	Noble et al., 2002; Whetstone et al., 2003; Yu et al., 2008b
	MMP-12	Critical for migration of blood-borne macrophages into inflammatory site; MMP-12 knockouts showed reduced microglial/macrophage density and permeability of the blood–spinal cord barrier, and significantly improved functional recovery	Wells et al., 2003
Cysteine proteases	Calpains	Caused necrotic and apoptotic cell death; degraded cytoskeletal and other proteins and thereby lead to neurodegeneration; associated with reactive astrogliosis and inflammation; Calpain inhibitors inhibited neurodegeneration, extended survival, and improved motor function	Banik et al., 1998; Schumacher et al., 2000; Shields et al., 2000; Ray et al., 2001,2003a,2003b; Arataki et al., 2005; Yu and Geddes, 2007; Sribnick et al., 2007; Yu et al., 2008a
	Caspases	Induce apoptosis in neurons and glial cells; caspase inhibitors reported to mitigate injury-induced programmed cell death	Lou et al., 1998; Springer et al., 1999; Li et al., 2000a; Nottingham et al., 2002; Casha et al., 2005; Knoblach et al., 2005; McEwen and Springer, 2005; Adjan et al., 2007; Dasari et al., 2007a; Genovese et al., 2008
Aspartyl proteases	Cathepsin-B	May potentially be involved in degradation of myelin basic protein and the secondary injury cascade	Banik et al., 2000; Ellis et al., 2004
	Cathepsin-D	Possible role in the phagocytosis and lysosomal activation of microglia/macrophages	Moon et al., 2008
Serine proteases	uPA	uPA knockouts failed to show the structural remodeling of phrenic motor neuron synapses and the associated synaptic plasticity (SP); SP is required for the recovery of respiratory function following cervical SCI	Minor and Seeds, 2008; Seeds et al., 2011
	tPA	Reduced activation of microglia/macrophages and apoptotic cell death and improved functional recovery reported in tPA knockouts	Abe et al., 2003
	Granzyme-B Neuropsin	Involvement in neuronal death Attenuated demyelination, oligodendrocyte death, and axonal damage, and improved functional recovery reported in neuropsin knockouts	Chaitanya et al., 2009 Terayama et al., 2007

MMP, matrix metalloproteinase; ECM, extracellular matrix; CSPG, chondroitin sulfate proteoglycan; SCI, spinal cord injury; uPA, urokinase plasminogen activator; tPA, tissue plasminogen activator.

and a catalytic domain containing three histidines that coordinate a zinc atom (Birkedal-Hansen et al., 1993). The C-terminal of MMP molecules contains a hemopexin domain that is involved in anchoring to the ECM or cell surface and substrate recognition (Allan et al., 1991; Brooks et al., 1996; Gohlke et al., 1996; Murphy and Knauper, 1997; Overall, 2002). These MMPs are released as inactive zymogens that become active only when the propeptide is cleaved

(Chakraborti et al., 2003). Each member has specificity for a subset of ECM molecules. They are constitutively expressed and can degrade all components of the ECM and thus were named MMPs. MMPs are tightly associated with ECM turnover, which plays an active role in various physiological and pathological states. By affecting the proteolytic degradation or activation of the cell surface and ECM proteins, MMPs can modulate both cell-cell and cell-ECM interactions. Cell

surface receptor shedding, cytokine activation, chemokine inactivation, the release of apoptotic and antiapoptotic signal regulation for angiogenesis, and immune surveillance, are examples of molecular events mediated by MMPs. In brain and spinal cord injuries, MMPs have been shown to degrade components of the basal lamina, leading to disruption of the blood–brain barrier (BBB; Noble et al., 2002; Yong et al., 2007; Zhang et al., 2010), and contribute to oxidative stress (Yu et al., 2008b), demyelination (Zhang et al., 2010), leukocyte trafficking, and a progressive neuroinflammatory response (Rosell and Lo, 2008; Yong et al., 2007; Zhang et al., 2010).

In order to prevent inappropriate tissue injury, the regulation of MMPs occurs at three levels: (1) the level of transcription; (2) the activation of the zymogen; and (3) the inactivation of MMPs by their natural inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs; Chakraborti et al., 2003; Crocker et al., 2004). An imbalance between the active enzymes and their natural inhibitors leads to the accelerated destruction of connective tissue, which is associated with several pathologic processes, including cancer and arthritis. There are four known mammalian TIMPs: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. The N-terminal domain of TIMP proteins contains the MMP inhibitory domain, whereas the C-terminal domain of the TIMPs mediates important protein–protein interactions, in particular with the hemopexin domains of pro-MMPs (Crocker et al., 2004). In whole-brain homogenates from healthy adult specimens, TIMP-4 levels are most abundant, followed by TIMP-2 and TIMP-3, with TIMP-1 showing the lowest overall expression. TIMP-4 is widely expressed throughout the central nervous system (CNS; Young et al., 2002), while TIMP-3 expression is detected in the cerebral cortex, cerebellum, and thalamus (Vaillant et al., 1999). TIMP-2 mRNA is strongly expressed in healthy neurons in all regions of the CNS (Pagenstecher et al., 1998). TIMP-1 immunoreactivity is observed around the soma of Bergmann glia and in the Purkinje cell layer (Vaillant et al., 1999). The TIMPs differ in their affinity for specific MMPs. TIMP-1 and TIMP-2 are the predominant inhibitors of MMP-9 (gelatinase B), and MMP-2 (gelatinase A), respectively (Sternlicht and Werb, 1999).

Recent SCI investigations have demonstrated the involvement of MMPs in post-traumatic events (Hsu et al., 2006; Noble et al., 2002; Wells et al., 2003). MMPs support the infiltration of inflammatory cells into the injured spinal cord, and most likely contribute to early disruption of the blood–spinal cord barrier (BSCB), which is supported by the finding that the broad-spectrum MMP inhibitor BB-3103 decreases endothelial gap formation and occludin loss (Reijerkerk et al., 2006). As leukocytes transmigrate across the vascular wall they release MMPs, which in turn degrades tight-junction-related proteins and the surrounding basal lamina. Basal lamina proteins, such as fibronectin, laminin, and heparan sulfate, are degraded by MMPs (Rosenberg and Yang, 2007). Zonula occludens-1, VE-cadherin, and occludin are the substrates for several MMPs (Asahi et al., 2001; Buhler et al., 2009; Caron et al., 2005; Yang et al., 2007). In addition to the disruption of BSCB, oxidative stress contributes to pathogenesis in the injured spinal cord (Zhang et al., 2010). MMPs are regulated by reactive oxygen species, including nitric oxide and hypochlorous acid (Alexander and Elrod, 2002; Haorah et al., 2007). Transgenic animals that overexpress the antioxidant enzyme superoxide dismutase 1 showed

enhanced neuroprotection after SCI (Sugawara et al., 2002). Contrary to the damage they cause after SCI, MMPs support the recovery process by limiting the formation of an inhibitory glial scar and degrading the inhibitory proteins, as well as cleaving extracellular proteins that sequester growth factors (Pizzi and Crowe, 2007; Yong et al., 2001).

Upregulation of mRNA transcripts encoding MMP-3, MMP-7, MMP-9, MMP-10, MMP-11, MMP-13, MMP-19, and MMP-20, within 24 h of injury was reported in a murine model of SCI, whereas increased expression of MMP-2, MMP-12, and MMP-13 was delayed in onset until 5 days after injury (Wells et al., 2003). Recently we also have reported a similar trend in a rat spinal cord contusion model (Veeravalli et al., 2009a). MMP-1 expression was upregulated 1 day post-SCI (Veeravalli et al., 2009a; Xu et al., 2001). During its peak expression in the acutely-injured spinal cord, MMP-1 was localized to neurons and glia (Xu et al., 2001). Real-time polymerase chain reaction (PCR), zymography, and Western blots revealed a gradual rise in MMP-2 (gelatinase A), a predominant gelatinase in the injured spinal cord implicated in wound healing processes, including remodeling of the ECM and glial scar formation, which then remained elevated for weeks thereafter (Duchossoy et al., 2001; Goussev et al., 2003; Hsu et al., 2006; Veeravalli et al., 2009a; Xu et al., 2001). MMP-2 localizes to reactive astrocytes and neurons in the chronically injured cord (Hsu et al., 2006; Veeravalli et al., 2009a). Chondroitin sulfate proteoglycans (CSPGs), such as neurocan and versican, are degraded by MMP-2 (Pizzi and Crowe, 2007). By degrading CSPG and other inhibitory molecules, MMPs support axonal regenerative potential in the injured CNS (Pizzi and Crowe, 2007; Yong, 2005). MMP-2-null mice show greater CSPG immunoreactivity, fewer serotonergic fibers caudal to the injury site, and significantly reduced motor recovery, compared with wild-type mice after a contusive SCI (Hsu et al., 2006). Zymography and Western blots revealed a transient increase in MMP-9 at 1 day post-injury (Duchossoy et al., 2001; Goussev et al., 2003; Xu et al., 2001; Yu et al., 2008b). MMP-9 is a key mediator of early pathogenesis after SCI. MMP-9 is localized to neurons and glia during its peak expression in the acutely-injured spinal cord (Xu et al., 2001). MMP-9 is also detected in blood vessels, neutrophils, and macrophages (Noble et al., 2002; Xu et al., 2001; Yu et al., 2008b). Immunological depletion of neutrophils prior to SCI resulted in decreased MMP-9 activity in the injured spinal cord, suggesting that these leukocytes are the principal source of MMP-9 in the injured tissue (de Castro et al., 2000). MMP-9 may contribute to the disruption of the barrier (Noble et al., 2002), aside from its ability to kill neurons (Xue et al., 2006). Barrier disruption to the protein luciferase is maximal at 24 h post-injury, a time point that corresponds to the peak activity of MMP-9 (Noble et al., 2002; Whetstone et al., 2003). In addition, barrier disruption is reduced in MMP-9-knockout mice, as well as normal mice treated with the broad-spectrum MMP inhibitor GM6001, that are treated early during the maximal expression period from 3 h to 3 days post-injury. Similarly to findings with GM6001, intrathecal administration of the selective gelatinase inhibitor SB-3CT 2 h before injury to the rat spinal cord reduces both MMP-9 activity and barrier disruption by 1 day post-injury and decreases apoptotic cell death (Yu et al., 2008b). Unlike wild-type animals, in the superoxide dismutase 1-transgenic animals, there was no increase in active MMP-9 at days 1, 3, and 7 after SCI, and a

decrease in barrier disruption and apoptosis (Yu et al., 2008b). Moreover, in MMP-9-knockout animals, glial scar formation was abrogated, along with reduced CSPG immunoreactivity at the lesioned epicenter after SCI (Hsu et al., 2008). Recent investigations revealed that melatonin, the pineal secretory hormone, offered protection against SCI in mice not only by its anti-inflammatory activity, but also by reducing the activity and expression of MMP-2 and MMP-9 (Esposito et al., 2008). Further, administration of valproic acid, a histone deacetylase inhibitor, after SCI inhibited MMP-9 activity, attenuated BSCB permeability, reduced TNF- α expression, inhibited apoptotic cell death and caspase-3 activation, reduced lesion volume, and improved functional recovery (Lee et al., 2012). Similarly to other MMPs, temporal and cellular expression of MMP-12 varies according to the type and severity of SCI. Increased expression of MMP-12 was delayed in onset until 5 days after injury (Wells et al., 2003). Our recent studies demonstrated the most marked upregulation of MMP-12 on day 21 (a 1737-fold increase compared to control values), which is consistent with the earlier published data in mice, wherein the authors reported a 189-fold increase in MMP-12 expression from basal levels on day 5 (Veeravalli et al., 2009a; Wells et al., 2003). MMP-12 is expressed primarily in microglia/macrophages (Wells et al., 2003). MMP-12 is critical for the migration of blood-borne macrophages across the endothelial basement membranes into inflammatory sites (Shipley et al., 1996). It is likely that MMP-12 influences the migration of macrophages into the injured spinal cord. Comparisons of cell density of Iba-1-positive elements reveal fewer macrophages and microglia in MMP-12-null mice compared to wild-type animals (Wells et al., 2003). Spinal cord injury in MMP-12-null animals reduced the permeability of the BSCB and significantly improved functional recovery (Wells et al., 2003). In support of a pathogenic role for MMP-9 and MMP-12 in SCI, MMP-9- and MMP-12-knockout mice showed marked improvement in functional recovery compared to wild-type controls. Conversely, MMP-2 levels increased between 7 and 14 days after SCI, and MMP-2-knockout mice did not recover as well from the injury (Hsu et al., 2006).

The SCI results show an extensive upregulation of several MMP members in the early periods after SCI, and a more restricted expression in the later periods post-trauma. Further, it has become clear that MMPs have differing roles in both pathogenesis and recovery after SCI. The multiple roles of MMPs after SCI make the therapeutic targeting of MMP activity a challenge. Pharmacological blockade of MMPs limited to the first 3 days after injury is neuroprotective and promotes long-term recovery of hindlimb function (Noble et al., 2002). This gain of function is lost, however, if blockade of MMPs is extended to the first week after injury (Trivedi et al., 2005). In general, given a massive upregulation of several MMPs after SCI, it is rational to use potent and broad-spectrum MMP inhibitors in the early periods after the trauma. The treatment duration will have to be limited, given the evidence that MMPs subsequently attempt some degree of repair. Therefore, the application of broad-spectrum MMP inhibitors in the more chronically-injured spinal cord should be approached with caution because untoward effects may outweigh any benefit.

Cysteine proteases

Cysteine proteases have profound involvement in apoptosis, inflammation, and abnormal immune responses in

human disorders. Abnormal activation of these proteases plays an important role in the pathogenesis of both acute and chronic SCI. Calpain is the most important cysteine protease for neurodegeneration in SCI. Other cysteine proteases, such as caspases and cathepsins, also make contributions to neurodegeneration in SCI. Caspases are the main cysteine proteases for execution of apoptosis, and these are also activated for apoptotic death of neurons and glial cells in SCI (Ray et al., 2003a). Increased activity of these cysteine proteases degrades cytoskeletal proteins and other survival factors, leading to apoptosis of CNS cells in the progression of secondary injury after SCI.

Calpains are a family of calcium-dependent cysteine proteases (Ray and Banik, 2003). Increased calpain activity has been implicated in the pathogenesis of various CNS injuries, including SCI (Ray and Banik, 2003). Calpains play prominent roles in causing both necrotic and apoptotic death after SCI (Ray et al., 2001; Ray et al., 2003a). They exist as proenzymes in two isoforms, milli-calpain (m-calpain) and micro-calpain (μ -calpain). After their activation by either reactive oxygen species or increased intracellular free calcium concentrations, these proenzymes degrade cytoskeletal and other proteins and thereby lead to neurodegeneration after SCI (Banik et al., 1998; Schumacher et al., 2000; Yu and Geddes, 2007). Calpains are also associated with reactive astrogliosis and inflammation after SCI, and thus their inhibition can control these detrimental processes (Ray et al., 2001; Shields et al., 2000). Therapeutic use of calpain inhibitors to some extent provided significant neuroprotection, with extended survival and preservation of motor function (Ray et al., 2003a; Yu et al., 2008a). Calpain inhibitors inhibited neurodegeneration and improved motor function after SCI in animal models (Arataki et al., 2005; Ray et al., 2003b; Sribnick et al., 2007; Yu et al., 2008a). Calpain has been proposed to work upstream of another cysteine protease, caspase-3, for induction of apoptosis after SCI (Ray et al., 2001). Repeated injections of melatonin in rats within 4 h after SCI reduced the content of thiobarbituric acid-reactive substances and myeloperoxidase activity, and facilitated the recovery of damaged spinal cord (Fujimoto et al., 1976). In another study, melatonin decreased the expression and activity of calpain and caspase-3, and thereby attenuated inflammation, axonal damage, and neuronal death in animals after SCI (Samantaray et al., 2008).

The interleukin-1 β -converting enzyme (ICE) family now consists of 11 members (Boldin et al., 1996; Duan et al., 1996a,1996b; Faucheu et al., 1995; Fernandes-Alnemri et al., 1995a,1995b,1996; Kamens et al., 1995; Kumar et al., 1994; Lippke et al., 1996; Munday et al., 1995; Muzio et al., 1996; Tewari and Dixit, 1995; Wang et al., 1994,1996), which can be divided into three subfamilies: the ICE subfamily, the CPP32 subfamily, and the Ich-1 subfamily, which have been named caspases (Alnemri et al., 1996). Caspases are another important class of cysteine proteases that are crucial effectors in cell death-signaling pathways (Jin and el-Deiry, 2005). ICE-like proteases promote the programmed cell death of neurons induced by trophic factor deprivation *in vitro* (Gagliardini et al., 1994; Li et al., 1996). Numerous studies have demonstrated the presence of multiple caspases and apoptosis following SCI (Beattie et al., 2000; Citron et al., 2000; Crowe et al., 1997; Eldadah and Faden, 2000; Keane et al., 2001; Liu et al., 1997; Lou et al., 1998; Springer et al., 1999; Yong et al., 1998). All caspases are translated initially as inactive zymogens, that

are then activated after specific cleavage, and have the following structural features in common: an N-terminal prodomain of variable length (22 to >200 amino acids), a large subunit (~17–20 kDa), a short inter-subunit region (~10 amino acids), and a small subunit (~10–12 kDa). The C-terminal portion of the large subunit contains the catalytic cysteine residue. Flanking this are other conserved residues that together form the semi-conserved pentapeptide sequence QACXG at the active site (Alnemri et al., 1996; Thornberry and Lazebnik, 1998). Pro-caspases are processed by limited proteolysis into their active form, which consists of a large- and small-subunit dimer. *In vivo*, however, caspases are more conformationally stable as tetramers consisting of two large/ small-subunit dimers (Eldadah and Faden, 2000). Once activated by specific cleavage to active forms, caspases can activate other pro-caspases via the extrinsic pathway directly, or the intrinsic pathway by mitochondrial-dependent mechanisms, thereby amplifying the programmed cell death process (Li et al., 1998; Scaffidi et al., 1998; Slee et al., 1999; Yakovlev and Faden, 2001; Yu et al., 2009). Based on their putative functions and sequence homologies, caspases are often categorized into three groups: apoptotic initiators (caspase-2, caspase-8, caspase-9, and caspase-10), apoptotic executioners (caspase-3, caspase-6, and caspase-7), and inflammatory mediators (caspase-1, caspase-4, caspase-5, caspase-11, caspase-12, and caspase-13); (Alenzi et al., 2010; Thornberry and Lazebnik, 1998).

Extensive studies have already confirmed the activation of caspases of both the extrinsic and intrinsic pathways for mediation of apoptosis in neurons and glial cells after acute and chronic SCI (Adjan et al., 2007; Casha et al., 2005; Dasari et al., 2007a; Genovese et al., 2008; Knobloch et al., 2005). The receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway reunite at the final phase of apoptosis for activation of caspase-3, which cleaves the inhibitor of caspase-activated DNase (CAD) for activation and translocation of CAD to the nucleus for fragmentation genomic DNA (Ray et al., 2003a). Caspase-3, the final executioner of apoptosis, has been found to be activated for apoptosis of neurons and glial cells in animal models of SCI (McEwen and Springer, 2005; Nottingham et al., 2002). The involvement of caspase-3 as a major effector in injury-induced neuronal apoptosis was established by using specific caspase inhibitors in various models of ischemic or traumatic injury (Clark et al., 2000; Gillardon et al., 1997; Gottron et al., 1997; Namura et al., 1998; Yakovlev et al., 1997). Blocking ICE-like protease activity delays motoneuron death induced *in vitro* by trophic factor deprivation, and *in vivo* during development (Milligan et al., 1995). Obviously, inhibition of caspases is an essential goal for functional neuroprotection in SCI (Festoff et al., 2006; Okutan et al., 2007). Several SCI experiments using caspase inhibitors have been reported to mitigate injury-induced programmed cell death (Li et al., 2000a; Lou et al., 1998; Springer et al., 1999). Caspase inhibition prevents neuronal damage, and by so doing, preserves and protects neurological function. Therefore the inhibition of caspases could improve locomotor function, and thereby offer novel treatments for SCI in humans. However, caspase inhibition has yet to be used in the clinical setting despite demonstrated efficacy in the treatment of various CNS insults in *in vivo* models (Braun et al., 1999; Hara et al., 1997; Li et al., 2000b; Yakovlev et al., 1997).

Another class of cysteine proteases, cathepsins, can also play an important role in the regulation of apoptosis (Conus and Simon, 2008; Ivanova et al., 2008; Stoka et al., 2007). The contribution of these lysosomal acidic proteases to neurodegeneration after SCI is not yet well recognized. However, few investigations suggest that cathepsins also participate in the pathogenesis of SCI, and inhibition of cathepsins can be an important strategy to enhance neuroprotection after SCI (Banik et al., 1986,2000). There are more than a dozen cathepsins, which are mostly cysteine proteases, and only a few are aspartyl and serine proteases. Cathepsin B (a cysteine protease), and cathepsin D (an aspartyl protease), are known to cause pathogenesis in SCI (Banik et al., 1986). Cathepsin B activation and increased expression levels occurred at the site of injury, as well as in the segments rostral and caudal to the site of injury following SCI (Ellis et al., 2004). Localization of cathepsin D, mostly in activated macrophages and microglia in the compression lesions after SCI, suggest a possible role of cathepsin D in the phagocytosis and lysosomal activation of macrophages and microglia (Moon et al., 2008). Activated microglia may also secrete cathepsin B for promotion of neuronal apoptosis (Kingham and Pocock, 2001). Cathepsins and caspases can be considered to be collaborators for the mediation of apoptosis (Turk and Stoka, 2007). The cleavage of Bid to tBid, and degradation of anti-apoptotic Bcl-2 proteins by the lysosomal cathepsins, are supposed to be the links to the release of cytochrome c from mitochondria to cytosol for eventual activation of caspase-3 (Droga-Mazovec et al., 2008; Reprnik and Turk, 2010).

Serine proteases

The two plasminogen activators in mammals, urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), are serine proteases. The plasminogen activator/plasminogen proteolytic cascade is known for its role in thrombolysis (Collen, 1999,2001). Plasminogen activators are best known as “clot busters,” when they activate the proenzyme plasminogen to the broad-acting and fibrin-degrading protease plasmin in the vascular system (Collen, 1980). The function of plasminogen activators that convert the zymogen plasminogen to the active protease plasmin (Collen and Lijnen, 1991), however, is not limited to the initiation of thrombolysis. Plasminogen activators also play important roles in other tissues, where they have been shown to promote cellular remodeling associated with a number of physiological events, including angiogenesis, ovulation, trophoblast implantation, bone growth, muscle differentiation, and tumor cell metastasis (Dano et al., 1999). In addition, plasminogen activators activate other proenzymes such as MMPs (Baramova et al., 1997; Keski-Oja et al., 1992; Siconolfi and Seeds, 2003). The presence of plasminogen and plasminogen activator inhibitor-1 in the brain indicates involvement of the plasminogen activator/plasminogen cascade in the CNS (Masos and Miskin, 1997; Salles and Strickland, 2002; Teesalu et al., 2004; Yepes and Lawrence, 2004). In the nervous system, plasminogen activators play an active role in neural development, where they are secreted by both central and peripheral nervous system neurons to facilitate neuronal cell migration and axonal outgrowth (Friedman and Seeds, 1995; Krystosek and Seeds, 1981; McGuire and Seeds, 1990; Pittman, 1985; Seeds et al., 1999; Verrall and Seeds, 1989). In

addition, they can directly activate pro-neurotrophic factors, including the motor neuron survival factor hepatocyte growth factor (Mars et al., 1993; Thewke and Seeds, 1996), or indirectly via plasmin activation of pro-BDNF (brain-derived neurotrophic factor) and pro-NGF (nerve growth factor) to their active forms (Pang et al., 2004). Plasminogen activators also play an active role in dendritic spine formation (Oray et al., 2004), and have been implicated in synaptic remodeling associated with cerebellar motor learning, visual cortex ocular dominance columns, and both hippocampal and corticostriatal long-term potentiation (Baranes et al., 1998; Huang et al., 1996; Mataga et al., 2004; Muller and Griesinger, 1998; Seeds et al., 1995,2003).

Plasminogen activators are being upregulated to promote axonal regeneration following nerve injury (Hayden and Seeds, 1996; Nakajima et al., 1996; Siconolfi and Seeds, 2001a,2001b). In addition, plasminogen activators play an active role in synaptic plasticity associated with the crossed phrenic phenomenon (CPP). The CPP is one of the most dramatic examples of spinal cord plasticity, resulting in the recovery of respiratory function following a high cervical SCI (Goshgarian, 2003). CPP occurs in several mammalian species, including the mouse (Minor et al., 2006), following a cervical C2 spinal cord hemisection, which paralyzes the ipsilateral hemidiaphragm by interrupting the descending flow of respiratory impulses from the medulla to phrenic motor neurons. The induction of uPA was seen within 1 h in ipsilateral phrenic motor neurons, reaching maximal levels by 6 h, and disappearing by 20 h post-hemisection. Similarly, tPA levels increased in both phrenic motor neurons and Neu-N-positive interneurons within the phrenic motor nucleus; however, elevated tPA levels were still seen 20 h post-hemisection (Minor and Seeds, 2008). Thus, PA induction was concomitant with the critical latent period in the recovery of diaphragmatic function during the CPP. Knockout mice lacking different genes in the plasminogen activator/plasmin system demonstrated that expression of uPA is required during the critical 1- to 2-h delay period following C2 hemisection for acquisition of a good CPP response (Minor and Seeds, 2008). uPA-knockout mice fail to show the structural remodeling of phrenic motor neuron synapses that underlie the CPP response (Minor and Seeds, 2008; Seeds et al., 2011). Further, uPA acts in a cell-signaling manner via binding to its receptor uPAR rather than as a protease (Seeds et al., 2011). The expression of uPA receptors uPAR and LRP-1 (the LDL-like receptor protein), are both upregulated in the ipsilateral phrenic motor nucleus following C2 hemisection, and may be targets for uPA-mediated cell signaling. Seeds and colleagues discussed the potential cell signaling pathways downstream of the uPA interaction with these cell surface receptors in the phrenic motor nucleus (Seeds et al., 2009). Knowledge of these uPA-mediated signaling pathways may identify potential means for pharmacological activation of the synaptic plasticity required for the recovery of phrenic motor neuron activity.

tPA plays both harmful and beneficial roles. After peripheral nerve injury, studies have shown that tPA fibrinolytic activity results in a reduction of axonal degeneration and demyelination (Akassoglou et al., 2000). In addition, tPA activity has been shown to promote axonal regeneration and functional recovery (Siconolfi and Seeds, 2001a,2001b). A neuroprotective effect of tPA against zinc-induced neuronal

death has also been reported (Kim et al., 1999). In contrast to these beneficial effects, plasmin, which is generated by the action of tPA on plasminogen, is thought to be involved in demyelinating diseases by degrading myelin basic protein (Cammer et al., 1978). Furthermore, tPA also contributes to excitotoxic neuronal death by activating microglia (Rogove et al., 1999; Tsirka et al., 1995), or enhancing NMDA-receptor-mediated signaling (Nicole et al., 2001). tPA may act through several possible mechanisms: (1) by promoting demyelination through plasmin, which directly degrades myelin basic protein (Cammer et al., 1978), and initiates the MMP activation cascade which has been documented to have an important role in the breakdown of myelin membranes (Cuzner and Opendakker, 1999); (2) by altering inflammatory reactions in the CNS by increasing the permeability of the BBB (Paterson et al., 1987); (3) by promoting excitotoxic cell death by contributing to glutamate-induced oligodendrocyte injury and neuronal death (Pitt et al., 2000; Smith et al., 2000); (4) by aiding neuronal regeneration by reducing local fibrin deposition (Akassoglou et al., 2000; Herbert et al., 1996); or (5) by promoting migration of oligodendrocyte progenitors through the extracellular matrix (Uhm et al., 1998). Although the effect of tPA on the nervous system has attracted much attention, its role in the secondary pathogenesis seen after SCI is not well studied. There are numerous mechanisms of secondary injury, including impairment of spinal cord blood flow (Bingham et al., 1975); electrolyte changes (Young and Koreh, 1986); the release of excitotoxic amino acids (Faden and Simon, 1988; Wrathall et al., 1992), free radicals (Milvy et al., 1973), and proteases (Banik et al., 1986); neuronal necrosis, axonal fragmentation, and demyelination (Balentine, 1978; Balentine and Hilton, 1980); and activation of the apoptotic cascade (Crowe et al., 1997). The mechanisms of action of tPA strongly suggest the possible involvement of tPA in secondary pathogenesis after SCI. Decreased neural damage in tPA-deficient mice after SCI (Abe et al., 2003) suggests that tPA might be one of the important factors that impair hindlimb motor function and spinal cord conduction after injury. Our recent studies demonstrated the beneficial effects of treating spinal cord-injured rats with human umbilical cord blood-derived mesenchymal stem cells (Dasari et al., 2007b,2008). Stem cell treatment significantly reduced elevated tPA levels and activity in spinal cord-injured rats (Veeravalli et al., 2009b). Stem cells were shown to facilitate the process of remyelination after SCI by differentiating into oligodendrocytes, which secrete neurotrophic hormones (neurotrophin-3 and BDNF), and help in the synthesis of myelin basic protein (Dasari et al., 2007b). However, we cannot rule out the possibility that these stem cells may block the degradation of myelin basic protein by downregulating tPA levels in spinal cord-injured rats, which ultimately results in locomotor recovery after SCI. This hypothesis was supported by our results, showing that myelin basic protein levels after SCI were significantly reduced during the peak activity of tPA, but reverted to baseline after the reduction of tPA activity and levels (Veeravalli et al., 2009b).

Granzymes (granzyme-A and granzyme-B) are serine proteases stored in cytoplasmic granules within cytotoxic T cells and natural killer (NK) cells. During SCI, BSCB breakdown facilitates the infiltration of T cells, NK cells, macrophages, and other immune cells to the site of injury, thereby contributing to secondary pathogenesis after SCI. The

purpose of the granzymes released from cytotoxic T cells and NK cells is to induce apoptosis. Granzyme B initiates cell death either by disrupting mitochondrial integrity; by activating caspases, which in turn activates CAD, the enzyme that degrades DNA; or by cleaving key structural proteins in the nuclear membrane or cytoskeleton (Goping et al., 2003; Metkar et al., 2003; Sharif-Askari et al., 2001; Zhang et al., 2001). Recent SCI investigations reported the crucial role of granzyme-B in neuronal death after SCI (Chaitanya et al., 2009). The cross-talk between granzymes and caspases might amplify and accelerate the cell death process after SCI.

Kallikreins, which are serine proteases, serve a variety of physiological functions, including regulation of blood pressure, and promotion of neuronal health and the inflammatory response (Borgono et al., 2004; Borgono and Diamandis, 2004; Diamandis et al., 2004). Neuropsin (the KLK8 gene is the human analog), a kallikrein-like serine protease, is an extracellular trypsin-type protease with a relatively narrow spectrum of substrates (Shimizu et al., 1998). Neuropsin was found to be expressed constitutively in the neurons of the limbic system of the adult mouse brain (Chen et al., 1995; Shiosaka and Yoshida, 2000; Yoshida and Shiosaka, 1999). Traumatic, excitotoxic, and immunological injuries induced neuropsin expression in the area of the CNS surrounding the lesion (Terayama et al., 2004, 2005; Tomizawa et al., 1999). Neuropsin-knockout mice showed attenuated demyelination, oligodendrocyte death, and axonal damage, and improved functional recovery after SCI compared to wild-type mice (Terayama et al., 2007).

Conclusions

Here we discussed both the positive and negative roles of various proteases, such as matrix metalloproteinases, cysteine proteases (calpains, caspases, and cathepsins), and serine proteases (urokinase plasminogen activator, tissue plasminogen activator, granzymes, and neuropsin), and their expression after SCI. Further, it has become clear that these proteases have differing roles in both pathogenesis and recovery after SCI. Targeting the right proteases at the right time would improve functional outcomes after SCI. In this scenario, using a non-specific protease inhibitor, or targeting a single protease for the treatment of injured spinal cords will likely not result in the desired therapeutic outcome. Therefore further studies are warranted to evaluate the effects of simultaneous inhibition of key proteases on functional outcomes after spinal cord injury.

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